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14. ABSTRACT
The long term goals of this research are to understand the mechanisms by which NF1 and its partners control growth using the Drosophila peripheral nerve as our assay system. This system is advantageous because we can apply a number of powerful molecular genetic methodologies that are not available in other systems. For task #3, we used RNA interference to generate preliminary evidence that push actsin the peripheral glia to control perineurial glial growth. This preliminary finding will be pursued in much greater detail during the next funding period. Our major findings continue to be generated from aim #4. Our finding, reported last year, that Ras nonautonomously activates perineurial glial growth via PI3 Kinase, Akt and FOXO, was published in the Journal of Neuroscience. During this funding period we found evidence for a role for the Akt-regulated Tor pathway in regulating glial growth: for example, expression of a dominant-negative S6 kinase suppressed the increased glial growth conferred by PI3K-CAAX. This and other observations raise the possibility that Tor and FOXO interact to control glial growth. We also report evidence that the Ras-activated Ral GTPase participates with PI3K inglial growth control.

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INTRODUCTION

Over the last several years, my lab has been developing the Drosophila peripheral nerve as a system with which to identify and study the signalling pathways controlling growth of the perineurial (outer) glial layer. To accomplish this goal, we apply the various molecular genetic methodologies uniquely available in Drosophila; we hope that these methodologies will enable us ultimately to identify all of the relevant genes that interact with *NF1* to control growth, and place *NF1* and these partner genes in as complete a mechanistic context as possible. Then this mechanism could be tested and refined in systems more similar to humans but more difficult to work with (e.g. the mouse). Because all of the experiments are performed on the acutely dissected third instar larva, there are no complications or caveats associated with experimentation on cell culture systems, and we assay the entire nerve cross section as it exists within the whole organism. We thought that a more complete mechanistic understanding of growth control within peripheral nerves would greatly facilitate the ability to design drugs able to combat neurofibromas. Within this larger context, I proposed four different tasks to investigate various aspects of the genetic control of growth within peripheral nerves. These tasks involve elucidation of the relationship among Neurofibromin, pushover, and protein kinase A, as well as the identification of signalling pathways downstream of Ras that affect growth within peripheral nerves. For this funding period (months 25-36) I continued work on task #3. As was the case last year, our work on task #4 was by far the most successful, and therefore most of the efforts of the lab was concerned with extending these analyses.

BODY

Task three: Does *pushover (push)* act in the PKA pathway or a parallel pathway to control perineurial glial growth? I decided that it was essential to establish the cell type (neuronal, peripheral glia, or perineurial glia) in which *push* functions to control glial growth before performing any subsequent experiments. I had previously hypothesized that *push* was expressed and acting in the peripheral glia, not the perineurial glia, because the perineurial glia was reported to originate from the mesoderm, whereas *push* is not expressed in the mesoderm (at least during embryogenesis). However, we subsequently established that the perineurial glia expresses the glial marker *repo-Gal4*, which raises the possibility that the perineurial glia might actually be of glial origin after all, and thus might express *push*.

To identify the cell type in which *push* functions, we constructed a *push*-RNAi construct under *Gal4/UAS* control and analyzed perineurial glial thickness in larvae in which this construct was driven in motor neurons (using the *D42 Gal4* driver), in peripheral glia (using *gli-Gal4*) and in perineurial glia (using *repo-Gal4* - this driver is anticipated to express in peripheral glia as well) and compared these values to the value observed in the *push* null mutant. We found that *push*-RNAi driven by *D42* and *repo-Gal4* conferred perineurial glial thicknesses of 1.47 +/- 0.07 µm, n=37 and 1.49 +/- 0.07 µm, n=33, respectively. These values are not significantly different from wildtype. In contrast, *push*-RNAi driven by *gli-Gal4* conferred a perineurial glial thickness of 1.93 +/- 0.18 ¨m, n=19, which is significantly different from wildtype but not from the *push* null mutant (2.07 +/- 0.12 µm, n=24). Therefore, I conclude tentatively that *push* is acting in the peripheral glia to control glial growth.

In the next funding year, I propose additional experiments to test this conclusion. In particular, I propose to see if peripheral-glial expression of the *push*-RNAi construct enhances the effects on perineurial glial thickness of Ras^{V12} and null mutations in *inebriated*, as was reported previously. If we find obtain positive results from these experiments, as anticipated, we will go on and see if *push* regulates PI3K activity in peripheral glia. This analysis will be accomplished by using the nuclear/cytoplasmic localization of FOXO as a readout for PI3K activity (elevated PI3K activity causes FOXO to be excluded from the nucleus). In particular, we will use the available anti-FOXO antibody to

compare nuclear FOXO levels in peripheral glia from wildtype versus the *ine push* double mutant. Peripheral glial nuclei will be specifically marked by co-expression of a nuclear localized GFP under control of *gli-GAL4.*

Task four: Identification of additional Ras signalling components regulating perineurial glial growth. Last year I reported that the effects of Ras on promoting growth nonautonomously in peripheral nerves were mediated via effectors PI3 kinase (PI3K), the kinase Akt, and the transcription factor FOXO. These studies were published recently in the *Journal of Neuroscience* (Lavery et al., 2007). I have appended this manuscript to this report and will therefore not describe these studies in detail. The results shown in figure 6 of this paper demonstrated that FOXO overexpression was sufficient to block the ability of PI3K to activate perineurial glial growth. However, these results do not tell us if FOXO is the only target of PI3K relevant for glial growth control, or if PI3K regulates glial growth via target(s) in addition to FOXO.

To distinguish between these possibilities, we evaluated perineurial glial growth in the heteroallelic *FOXO* null combination $FOXO^{21}/FOXO^{25}$. We reasoned that if FOXO were the only relevant target for PI3K, then PI3K would increase perineurial glial growth solely by inactivating FOXO. If so, then inactivating FOXO by mutation would be predicted to increase perineurial glial thickness to at least the same extent as overexpressing PI3K-CAAX. In fact, we found that perineurial glial thickness was normal in $FOXO^{21}/FOXO^{25}$ (Figure 1), suggesting that PI3K-CAAX regulates glial growth via targets in addition to FOXO. Obvious candidates for these additional targets include members of the Tor pathway, which are also regulated by PI3K, and which were previously shown by the Cichowski and Gutmann labs to be critically involved in mediating *Nf1*-dependent growth signals in both mouse fibroblasts and astrocytes (DasGupta et al., 2005; Johannessen et al., 2005).

Figure 1:

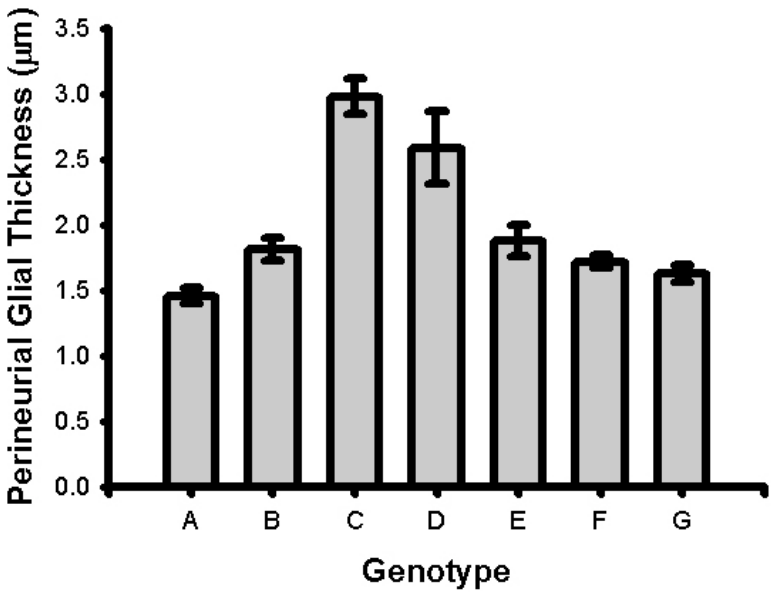

Figure 1: Evidence that PI3K acts through target(s) in addition to FOXO: Y Axis: Means +/- SEMS of perineurial glial thickness (µm) for the indicated genotypes (X Axis). Genotypes were as follows: A: *gli-Gal4/+*, n=60, B: *UAS-PI3K-CAAX/+*, n=59, C: *gli>PI3K-CAAX*, n=76, D: *gli>GFP, PI3K-CAAX*, n=25, E: *gli>PI3K-CAAX*, $S6Kinase^{DN}$, n=32, F: $gli>S6Kinase^{Act}$, n=26, G: $FOXO^{21}/FOXO^{25}$,n=24

To see if activation of a subset of the Tor pathway in peripheral glia was sufficient to increase perineurial glial growth, we measured glial thickness in larvae expressing activated *S6 kinase* in peripheral glia. We found that expressing this transgene caused no abnormal nerve growth phenotype (Figure 1), suggesting that activating this pathway is not sufficient to promote nonautonomous growth. However, we found that activity of this pathway is necessary for PI3K to promote nonautonomous growth: co-expression of the constitutively active *PI3K-CAAX* with a dominant-negative *S6 kinase* in

peripheral glia suppressed the increased glial growth observed with *PI3K-CAAX* expression alone (Figure 1). These results, taken together, raise the possibility that PI3K promote nonautonomous growth by activating S6 kinase and inhibiting FOXO. This possibility will be tested by introducing the *FOXO* null mutations into larvae expressing activated *S6 kinase* in peripheral glia and seeing if perineurial glial thickness is increased.

Our evidence that the Ral GTPase might be involved in nonautonomous growth regulation came from experiments performed for other reasons. We had previously demonstrated that activating Ras in peripheral glia promotes perineurial glial growth (Lavery et al., 2007), but we then wanted to know if Ras activity might also be required in the perineurial glia to respond properly to the growth promoting effects of the peripheral glia. To test this possibility, we introduced a heteroallelic loss of function combination of *Ras (Ras^{e2F}/Ras^{12A}*) into larvae expressing activated PI3K in the peripheral glia. We found that the *Ras* alleles significantly suppressed the growth promoting effect of activated PI3K (Figure 2). To show that Ras was exerting this effect in the perineurial glia, rather than the peripheral glia, we wanted to show that blocking Ras specifically in the peripheral glia (by expressing the dominant-negative *Ras^{N17}* transgene) was not able to suppress the growth-promoting effects of activated PI3K. But to our great surprise, we found that co-expression of *Ras^{N17}* completely suppressed the growth-promoting effects of PI3K (Figure 2). In contrast, co-expressing the constititutively-active *Ras$^{V`12}$* enhanced the growth-promoting of PI3K (Figure 3).

Figure 2:

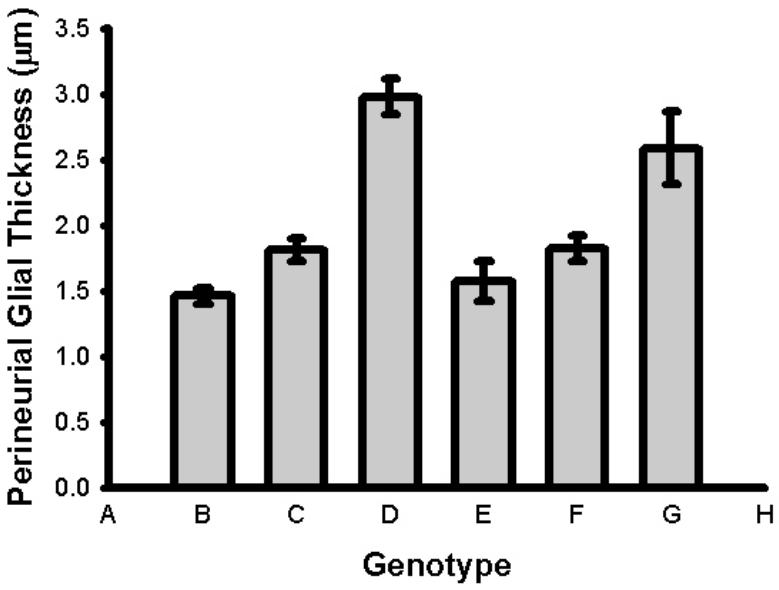

Figure 2: Ras activity in the peripheral glia is required for PI3K to increase perineurial glial growth. Y Axis: Means +/- SEMS of perineurial glial thickness (µm) for the indicated genotypes (X Axis). Genotypes were as follows: A: *gli-Gal4/+*, n=60, B: *UAS-PI3K-CAAX/+*, n=59, C: *gli>PI3K-CAAX*, n=76, D: *gli>PI3K-CAAX; Ras^{12A}/Ras^{e2F}*, n=30, E: *gli>PI3K-CAAX, Ras^{N17}*, n=31, F: *gli>PI3K-CAAX, GFP*, n=25.

Because PI3K-CAAX, the constitutively active allele that we used to promote growth is predicted to be Ras-independent, we thought it unlikely that Ras^{N17}was decreasing PI3K activity. Rather, we thought it was more likely that Ras^{N17} was blocking a second Ras-activated signalling pathway that was required for PI3K to induce growth. This second pathway did not appear to be the Raf-Erk pathway because co-expression of either a dominant-negative or constitutively-active *Raf* transgene with *PI3K-CAAX* had only moderate effects on perineurial glial thickness (Figure 3). The Ral-GTPase represents a third, Ras-activated pathway. Active Ras activates Ral by activating the Ral guanine dissociation factor.

To test the possibility that Ral is involved in the control of perineurial glial growth, we expressed the constitutively active *Ral^{V20}* transgene in peripheral glia and found no effect on perineurial glial

growth (Figure 3). However, expression of *Ral*^{V20} significantly enhanced the ability of PI3K-CAAX to promote perineurial glial growth (Figure 3), and this enhancement was approximately the same as the enhancement conferred by co-expression with *Ras*^{V12}. These results support the possibility that Ral activity regulates the ability of PI3K to promote peripheral nerve growth nonautonomously.

Figure 3:

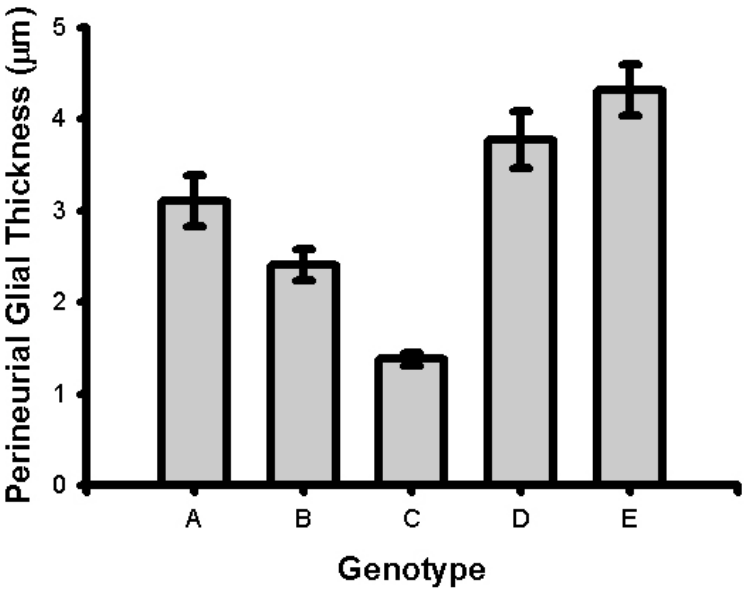

Figure 3: Ral, but not Raf potentiates the effects of PI3K on perineurial glial growth. Y Axis: Means +/- SEMS of perineurial glial thickness (µm) for the indicated genotypes (X Axis). Genotypes were as follows: A: *gli>PI3K-CAAX, Raf*^{gof}, n=52, B: *g.i>PI3K-CAAX, Raf*^{DN}, n=24, C: *gli>Ral*^{V20}, n=29, D: *gli>PI3K-CAAX, Ras*^{V12}, n=39, and E: *gli>PI3K-CAAX, Ral*^{V20}, n=30.

FUTURE GOALS FOR TASK #4;

First, we would like to see if perineurial glial growth is increased in a PI3K-independent manner if the Tor pathway is constitutively activated in the absence of FOXO protein (*FOXO*null background). We will activate the Tor pathway by expressing the transgene encoding *Tor*, an activated *S6 kinase*, a wildtype *eIF-4E*, or both *S6 kinase* and *eIF-4E*. All needed transgenes are present in the lab or available from the Drosophila stock center at Bloomington, IN.

Second, we would like to pursue the possibility that Ral is an essential co-activator of growth within peripheral nerves. Our next set of experiments, which should be complete in the next six weeks or so, will test the prediction that expression of *Ral*^{V20}, but not *Raf*^{gof}, will overcome the ability of *Ras*^{N17} co-expression to suppress the growth-promoting effects of *PI3K-CAAX*. If so, then this result will demonstrate that the suppression of glial growth by *Ras*^{N17} occurs by preventing activation of Ral.

It currently appears to be impossible to publish the phenotypic effects of transgene misexpression without including phenotypic analyses of chromosomal loss of function mutations, and often RNAi studies as well. This is because of a concern that any effects of transgene expression could be artifacts due to overexpression. Therefore, to bring mechanisms involving Ral to publication we will have to perform these experiments. Unfortunately, Ral is much less investigated than Raf or PI3K, and convenient genetic reagents are scarce; for example, no point mutations exist in this gene. However, there are a few deletions and transposon insertions that reduce Ral function and will be tested phenotypically. In addition, some of these insertions will be used to generate additional alleles if necessary.

KEY RESEARCH ACCOMPLISHMENTS

We report preliminary evidence that *push* acts in the peripheral glia to control perineurial glial thickness.

We published a paper (Lavery et al., 2007) describing the roles of PI3 Kinase and effectors Akt and FOXO in the nonautonomous regulation of perineurial glial growth.

We have obtained preliminary evidence suggesting that the Ras-activated Ral pathway might interact with the PI3 Kinase pathway to increase perineurial glial growth.

REPORTABLE OUTCOMES

Lavery, W., Hall, V., Yager, J.C., Rottgers, A., Wells, M.C. and Stern, M. (2007). Phosphatidyl inositol 3-Kinase and Akt nonautonomously promote perineurial glial growth in Drosophila peripheral nerves. *J. Neurosci* **27:** 279-288.

CONCLUSIONS

I tentatively conclude that *push* acts in the peripheral glia to control perineurial glial growth. Further experiments will test this conclusion definitively. We have completed and published our analysis demonstrating that Ras activates perineurial glial growth nonautonomously by inhibiting action of the transcription factor FOXO in a PI3K- and Akt-dependent manner.

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Johannessen CM, Reczek EE, James MF, Brems H, Legius E, CichowskI K (2005) The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proc Natl Acad Sci USA 102:8573-8578.

APPENDIX

1) Abstract of presentation to the NNFF Consortium on NF1 and NF2 entitled " FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth", by William Lavery, Michael Stern.

2) Manuscript by Lavery, W., Hall, V., Yager, J.C., Rottgers, A., Wells, M.C. and Stern, M. (2007). Phosphatidyl inositol 3-Kinase and Akt nonautonomously promote perineurial glial growth in Drosophila peripheral nerves. *J. Neurosci* **27:** 279-288.

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Abstract

FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth.
William Lavery, Michael Stern. Biochemistry and Cell Biology, Rice University, Houston, TX.

Drosophila peripheral nerves, structured similarly to their mammalian counterparts, comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We found that expression specifically within the peripheral glia of the constitutively active Ras^{V12} increases growth of the perineurial glial layer. This nonautonomous effect of RasV12 is mediated by activation of the downstream effector PI3 Kinase (PI3K) because expression within the peripheral glia of the activated *PI3K-CAAX* also increases perineurial glial growth, and because the growth-promoting effects of Ras^{V12} are suppressed by loss of function mutations in PI3K or by co-expression within the peripheral glia of the dominant-negative $PI3K^{D954A}$. The nonautonomous, growth-promoting effects of PI3K-CAAX are suppressed in a dose-dependent manner by loss of function mutations in *Akt*, the kinase downstream of *PI3K*, and are enhanced by co-expression within the peripheral glia of an Akt^{+} transgene. These observations suggest that PI3K exerts its effects via activation of Akt. Finally, we show that the growth-promoting effects of *PI3K-CAAX* are suppressed by co-expression within the peripheral glia of $FOXO^{+}$, a transcription factor that is inhibited by Akt-dependent phosphorylation. We conclude that Ras-PI3K-Akt activity in the peripheral glia promotes growth of the perineurial glia by inhibiting FOXO. In mammalian peripheral nerves, the Schwann cell releases several growth factors that can affect the proliferative and migratory properties of neighbors. Some of these factors are oversecreted in Schwann cells defective in *Nf1*. Our results raise the possibility that neurofibromas might be caused at least in part by a Ras-PI3K-Akt-dependent inhibition of FOXO within Schwann cells.

Cellular/Molecular

Phosphatidylinositol 3-Kinase and Akt Nonautonomously Promote Perineurial Glial Growth in *Drosophila* Peripheral Nerves

William Lavery, Veronica Hall, James C. Yager, Alex Rottgers, Michelle C. Wells, and Michael Stern
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Drosophila **peripheral nerves, structured similarly to their mammalian counterparts, comprise a layer of motor and sensory axons wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). Growth and proliferation within mammalian peripheral nerves are increased by Ras pathway activation: loss-of-function mutations in *Nf1*, which encodes the Ras inhibitor neurofibromin, cause the human genetic disorder neurofibromatosis, which is characterized by formation of neurofibromas (tumors of peripheral nerves). However, the signaling pathways that control nerve growth downstream of Ras remain incompletely characterized. Here we show that expression specifically within the *Drosophila* peripheral glia of the constitutively active Ras^{V12} increases perineurial glial thickness. Using chromosomal loss-of-function mutations and transgenes encoding dominant-negative and constitutively active proteins, we show that this nonautonomous effect of Ras^{V12} is mediated by the Ras effector phosphatidylinositol 3-kinase (PI3K) and its downstream kinase Akt. We also show that the nonautonomous, growth-promoting effects of activated PI3K are suppressed by coexpression within the peripheral glia of $FOXO^{+}$ (forkhead box O) a transcription factor inhibited by Akt-dependent phosphorylation. We suggest that Ras–PI3K–Akt activity in the peripheral glia promotes growth of the perineurial glia by inhibiting FOXO. In mammalian peripheral nerves, the Schwann cell releases several growth factors that affect the proliferative properties of neighbors. Some of these factors are oversecreted in *Nf1* mutants. Our results raise the possibility that neurofibroma formation in individuals with neurofibromatosis might result in part from a Ras–PI3K–Akt-dependent inhibition of FOXO within Schwann cells.**

Key words: **neurofibromatosis; Ras; FOXO; cell growth; cell nonautonomy; Schwann cell**

Introduction

Peripheral nerves in both *Drosophila* and mammals contain an inner layer of motor and sensory axons surrounded by an inner peripheral glial layer (termed the Schwann cell in mammals) and an outer, mesodermally derived perineurial glia (termed the perineurium in mammals). Proper growth, development, and function of peripheral nerves require intercellular signaling among the cell types present. For example, formation of the perineurial sheath requires Desert Hedgehog secretion from Schwann cells (Parmantier et al., 1999). In addition, neurons and glia interact reciprocally to regulate function, at least in part through the release of, and response to, small molecule neurotransmitters (Colomar and Robitaille, 2004; Yuan and Ganetzky, 1999).

Individuals with the autosomal-dominant genetic disorder of neurofibromatosis, which is caused by mutations in *Nf1* (for review, see Cichowski and Jacks, 2001), form peripheral nerve tumors called neurofibromas at high frequency. Neurofibromas are thought to arise in individuals heterozygous for *Nf1* after spontaneous loss of the $Nf1^{+}$ allele within Schwann cells (Kluwe et al., 1999; Serra et al., 2000). *NF1* encodes a Ras GTPase activator and thus negatively regulates Ras. Although at least some of the growth deficits of $Nf1^{-}$ cells result from Ras hyperactivation, the Ras effector pathways mediating the various growth defects have not been fully characterized. It was reported recently that phosphatidylinositol 3-kinase (PI3K), Akt, and the Akt-dependent kinase Tor (target of rapamycin) are hyperactivated in *Nf1*-deficient mouse or human cells and that this activation was required for proliferation of tumor cells in culture (Dasgupta et al., 2005; Johannessen et al., 2005). These results are consistent with the well established role for the PI3K–Tor pathway in autonomous growth control (Hay and Sonenberg, 2004). However, there is much evidence that neurofibroma formation requires Schwann cell nonautonomous pathways (Sherman et al., 2000; Yang et al., 2003). For example, neurofibromas are heterogeneous at the cellular level and contain cell types that are not clonally related (i.e., Schwann cells and fibroblasts). This observation raises the possibility that neurofibroma formation requires the $Nf1^{-}$-dependent oversecretion of growth factors that

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increase the proliferation of heterozygous neighbors (Yang et al., 2003). The identity of the pathway(s) regulating nonautonomous growth has not been elucidated.

Here we use the *Drosophila* peripheral nerve to identify molecules acting within the peripheral glia that regulate growth nonautonomously. We find that expression of constitutively active Ras^{V12} specifically within the peripheral glia increases perineurial glial thickness. We also show that this nonautonomous, growth-activating effect is mediated by PI3K and Akt: PI3K and Akt activity within the peripheral glia are both necessary and sufficient to promote nonautonomous growth. Finally, we report that peripheral glial overexpression of *FOXO* (forkhead box O), which encodes a transcription factor inhibited by Akt-dependent phosphorylation and which antagonizes PI3K–Akt-dependent gene expression (Puig et al., 2003), suppresses the growth-promoting effects of activated PI3K. We conclude that the effect of Ras activity within the peripheral glia on perineurial glial growth is mediated by PI3K and Akt and suggest that this pathway promotes nonautonomous growth by inhibiting FOXO.

Materials and Methods

Drosophila *stocks, mutations, and crosses.* Gliotactin (*gli*)–*Gal4* and *MZ709* express *Gal4* in peripheral glia (Ito et al., 1995; Auld et al., 1995; Leiserson et al., 2000; Sepp and Auld, 1999) and were provided by Vanessa Auld (University of British Columbia, Vancouver, British Columbia, Canada) and Kei Ito (National Institute for Basic Biology, Okazaki, Japan), respectively; upstream activating sequence (*UAS*)–*PI3K–CAAX* and *UAS–PI3K*^{D954A} express a constitutively active and dominant-negative PI3K, respectively, under the transcriptional control of *Gal4* (Leevers et al., 1996) and were provided by Sally Leevers (Cancer Research Institute, London, UK); flies bearing *UAS–*Ras^{V12} (strong) on chromosome III, *UAS–*Ras^{+} (Lee et al., 1996; Karim and Rubin, 1998), *UAS–*Raf^{F179} (Brand and Perrimon, 1994), *UAS*–green fluorescent protein *(GFP)* nuclear localization signal *(nls)* (Shiga et al., 1996), and Akt^{4226} (Perrimon et al., 1996) were provided by the Bloomington *Drosophila* Stock Center (University of Indiana, Bloomington, IN). Two independent *UAS–Akt* transgenes (A. Park, personal communication to FlyBase) were provided by the Bloomington *Drosophila* Stock Center via Exelixis (South San Francisco, CA). *UAS–*Ras^{V12} (weak) on chromosome II (Karim and Rubin, 1998) was provided by Andreas Bergmann (M. D. Anderson Cancer Research Center, Houston, TX). *UAS–*Ral^{V20} (Sawamoto et al., 1999) was provided by Hideyuki Okano (Tokyo, Japan). Two independent *UAS–*$FOXO^{+}$ transgenes (Junger et al., 2003; Hwangbo et al., 2004) were provided by Marc Tatar (Providence, RI). Flies bearing two loss-of-function alleles of *PI3K*: $PI3K^{2H1}$ and $PI3K^{A}$ (Halfar et al., 2001), provided by Ernst Hafen (Zurich, Switzerland).

Standard *Drosophila* genetics techniques were used to establish the fly stocks and perform the crosses used in the experiments described. Because the *PI3K* and *Akt* loss-of-function alleles used confer either lethality or greatly reduced viability when homozygous, these alleles were maintained with balancers carrying the tubby *Tb* dominant marker, which can be scored in larvae. Third-instar larvae carrying the *Akt* or *PI3K* mutant alleles on both chromosomes, to be analyzed with electron microscopy, were recognized by their non-tubby appearance. For all experiments using either *Gal4* or *UAS* transgenes, the appropriate larvae were obtained after a cross of the *Gal4*-containing fly line to the *UAS*-containing fly line. Because *UAS–PI3K–CAAX* is located on the X chromosome, only female larvae heterozygous for these transgenes were analyzed. In all cases, larvae bearing the *Gal4* driver alone or the *UAS*-driven transgene alone were generated in parallel to the experimental larvae and used as controls.

Transmission electron microscopy. Larvae were grown to the wandering third-instar stage in uncrowded half-pint bottles at room temperature (22–23°C). Larvae were collected only during the first and second days after the initial third-instar larvae appeared. The dissections, fixations, and stainings were performed as described previously (Yager et al., 2001). Perineurial glial thickness was measured from the edge of the nerve to the axon-containing lumen and averaged from eight measurements made 12:00, 3:00, 6:00, and 9:00 and four positions in between. Measurements were not taken at positions in nerves in which a perineurial glial nucleus was encountered.

Fluorescence microscopy. Larvae were grown to the wandering third-instar stage as described above. These larvae were dissected with the protocol used for electron microscopy, except PBS was used for dissections. Dissected larvae were fixed in PBS containing 5% formaldehyde and 0.1% Triton X-100 for 15 min. Ventral ganglia and nerves were removed and placed in Vectashield (H-1000; Vector Laboratories, Burlingame, CA) containing a 1:1000 dilution of Hoechst stain (H-3570; Invitrogen, Carlsbad, CA). Nuclei were visualized with 4′,6′-diamidino-2-phenylindole and GFP filters on an Axioplan 2 epifluorescence microscope (Zeiss, Oberkochen, Germany) using MetaMorph software for micrograph acquisition (Molecular Devices, Palo Alto, CA).

Results

gli–Gal4 and *MZ709*: two *Gal4* drivers that express in the peripheral glia but not the perineurial glia.

Drosophila peripheral nerves contain a layer of ~80 motor and sensory axons, wrapped by an inner peripheral glia, which forms the blood–nerve barrier (Auld et al., 1995) and an outer, mesodermally derived perineurial glia (Edwards et al., 1993). A transmission electron micrograph (TEM) of a peripheral nerve cross section is shown in Figure 1*A*. Each peripheral nerve contains approximately eight peripheral glial nuclei (Sepp et al., 2000). In addition, each mm of peripheral nerve contains ~20 perineurial glial nuclei (W. Lavery and M. Stern, unpublished observations).

To evaluate the role of Ras signaling in nonautonomous growth control within peripheral nerves, we used the *Gal4/UAS* system (Brand and Perrimon, 1993) to express wild-type and mutant transgenes specifically within the peripheral glia. Two *Gal4* drivers, *gli–Gal4* and *MZ709*, were reported to express in the peripheral glia but not the neurons of peripheral nerves (Ito et al., 1995; Sepp and Auld, 1999; Leiserson et al., 2000; Sepp et al., 2000). The *gli–Gal4* driver is a particularly well characterized marker for peripheral glia. *gli–Gal4* was generated via gene conversion from a *gli–lacZ* enhancer trap line (Auld et al., 1995; Sepp and Auld, 1999), which was reported to express specifically in peripheral glia, exit glia, and some midline glia. The *gli–Gal4* driver was used to study peripheral glial dynamics during embryonic peripheral nerve development. This driver was also used to study peripheral glial anatomy during larval growth and at the mature third-instar larval neuromuscular junction and peripheral sensory structures (Sepp et al., 2000). These studies confirmed that *gli–Gal4* is expressed in peripheral glia but not motor and sensory neurons.

To confirm that *gli–Gal4* and *MZ709* do not express *Gal4* in the perineurial glia, we visualized the expression pattern of these drivers within peripheral nerves via induced expression of a nuclear-localized GFP. We also visualized the total complement of peripheral nerve nuclei (peripheral and perineurial glial) via the Hoechst DNA dye. As shown in Figure 1, *B* and *D*, there are ~20 nuclei per millimeter of peripheral nerve. Most of these are perineurial glial nuclei, whereas a few are peripheral glial nuclei. If *gli–Gal4* and *MZ709* express in the perineurial glia as well as peripheral glia, then we anticipate that, in *gli>GFP(nls)* and *MZ709>GFP(nls)*, most or all of these nuclei would contain GFP. In fact, as shown in Figure 1, *C* and *E*, we observe that only a few (presumably peripheral glial) nuclei from these larvae express GFP. Therefore, we conclude that neither *gli–Gal4* and *MZ709* expresses *Gal4* in the perineurial glia. We generally observe GFP in fewer than eight peripheral glial nuclei, which presumably results from cell-to-cell variability in *Gal4* expression levels, as

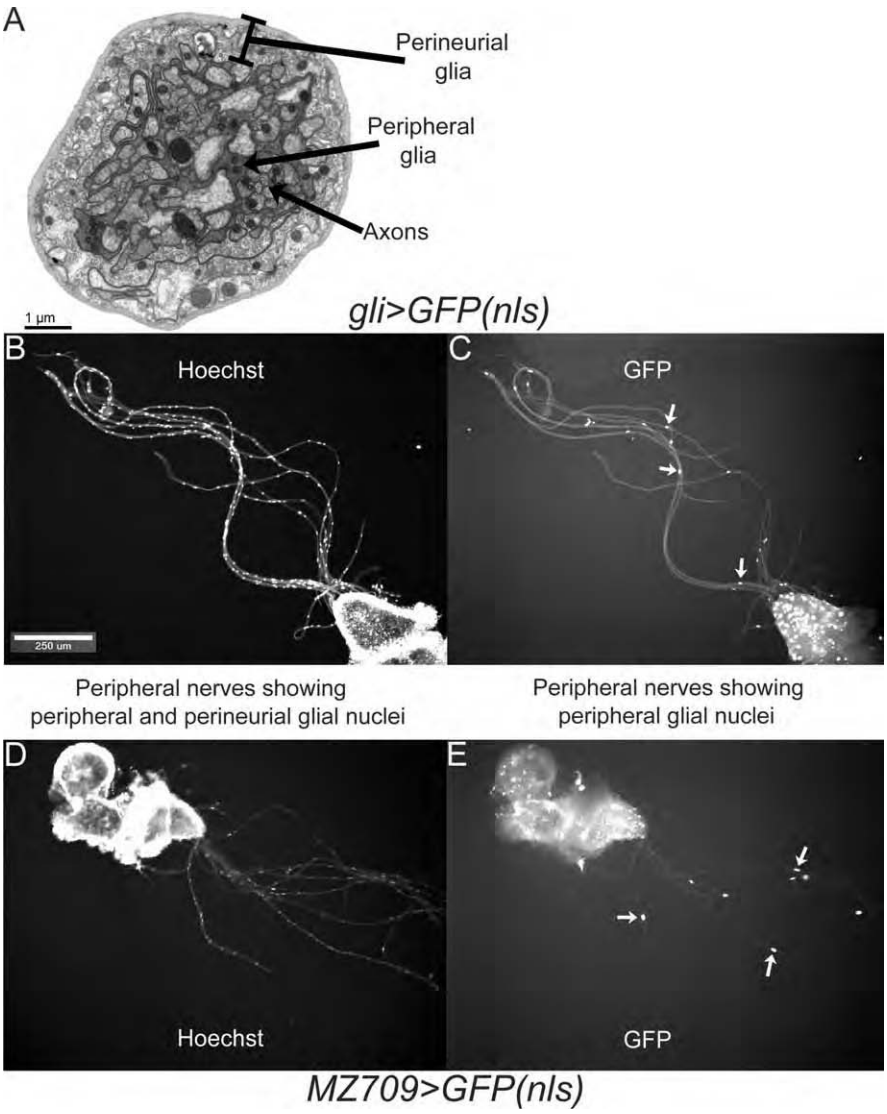


Figure 1. *gli–Gal4* and *MZ709* drivers are expressed in the peripheral glia but not the perineurial glia. ***A***, TEM of a cross section of a *gli>Ras*$^{+}$ third-instar larval peripheral nerve of wild-type thickness. The cell types present are indicated. ***B***, ***C***, Epifluorescence images of third-instar larval peripheral nerves from *gli>GFP(nls)* visualized for Hoechst and GFP, respectively. All nuclei (peripheral glial and perineurial glial) are visualized with the Hoechst stain (***B***), whereas only a few nuclei (presumed to be peripheral glial), some marked with arrows, are visualized with GFP (***C***). ***D***, ***E***, Same as ***B*** and ***C*** except that *MZ709>GFP(nls)* larvae were visualized. These observations demonstrate that *gli–Gal4* and *MZ709* are not expressed in perineurial glia.

was reported previously for peripheral glia (Sepp et al., 2001). We also observed that each driver also expresses *Gal4* within certain cells of the ventral ganglion (Fig. 1).

Expression of the constitutively active *Ras*^{V12} allele in peripheral glia increases perineurial glial growth

In both mice and humans, neurofibroma formation appears to occur only when the Schwann cell component of the peripheral nerve is homozygous for Nf1^{-} (Zhu et al., 2002; Kluwe et al., 1999). This observation suggests that activated Ras within Schwann cells is necessary for neurofibroma formation. To test the effects of activating Ras within *Drosophila* peripheral glia (analogous to the mammalian Schwann cell), we used the *gli–Gal4* driver to express *Ras*$^{+}$ or the constitutively active *Ras*^{V12} (Bourne et al., 1991; Lee et al., 1996; Karim and Rubin, 1998) specifically in the peripheral glia. We found that larvae bearing *gli–Gal4* and either of two *UAS–Ras*^{V12} transgenes exhibited a thickened perineurial glia. The thickness observed, 2.1–2.3 μm, was significantly (~50%) greater than the value observed in larvae carrying *gli–Gal4* or *UAS–Ras*^{V12} alone or *gli>Ras*$^{+}$ (Fig. 2). We conclude that Ras activation specifically within the peripheral glia is sufficient to promote perineurial glial growth. We also found that *gli–Gal4*-driven coexpression of both *UAS–Ras*^{V12} transgenes does not cause an additional increase in perineurial glial thickness: perineurial glial thickness in larvae expressing both transgenes is the same as in larvae expressing either transgene alone (Fig. 2). This observation suggests that, in *gli>Ras*^{V12} larvae, *Ras*^{V12} levels are not limiting for promoting perineurial glial growth. To rule out the possibility that the presence of two transgenes decreased expression of both via titration of *Gal4*, we measured perineurial glial thickness in larvae coexpressing *Ras*^{V12} with an indifferent transgene (*GFP*). We found that this coexpression did not suppress the growth-promoting effects of *Ras*^{V12} (Fig. 2), suggesting that the presence of a second *UAS*-driven transgene does not significantly affect expression of the first.

PI3K activation in the peripheral glia is sufficient to increase perineurial glial growth

Activated Ras activates a number of downstream molecules, including Raf, PI3K, and the guanine nucleotide exchange factor for the Ral GTPase (Kolch et al., 1991; Rodriguez-Viciana et al., 1994; Hofer et al., 1994). To identify the effector(s) responsible for transducing the nonautonomous growth activation conferred by *Ras*^{V12}, we expressed transgenes encoding the constitutively active *Raf*^{F179}, *PI3K–CAAX*, and *Ral*^{V20} proteins (Brand and Perrimon, 1994; Leevers et al., 1996; Sawamoto et al., 1999) within peripheral glia using *gli–Gal4*. As shown in Figure 3*B*, we found that expression of *Raf*^{F179} or *Ral*^{V20} had no significant effect on perineurial glial thickness. However, expression of *PI3K–CAAX* increased perineurial glial thickness to ~3 μm (Fig. 3*A*,*B*). This thickness is significantly greater than both wild-type thickness and the increased thickness conferred by *Ras*^{V12} expression. When *UAS–PI3K–CAAX* was expressed with a second peripheral glial driver, *MZ709* (Ito et al., 1995) (Fig. 1), perineurial glial thickness was increased to the same extent as with *gli–Gal4*. These results suggest that Ras exerts its nonautonomous effects on perineurial glial growth via activation of PI3K. The observation that *PI3K–CAAX* exerts a stronger effect than *Ras*^{V12} might indicate that PI3K levels are limiting in peripheral glia to promote perineurial glial growth. In this view, transgene-induced overexpression of *PI3K–CAAX* overcomes this limitation and enables a more robust growth effect to be observed.

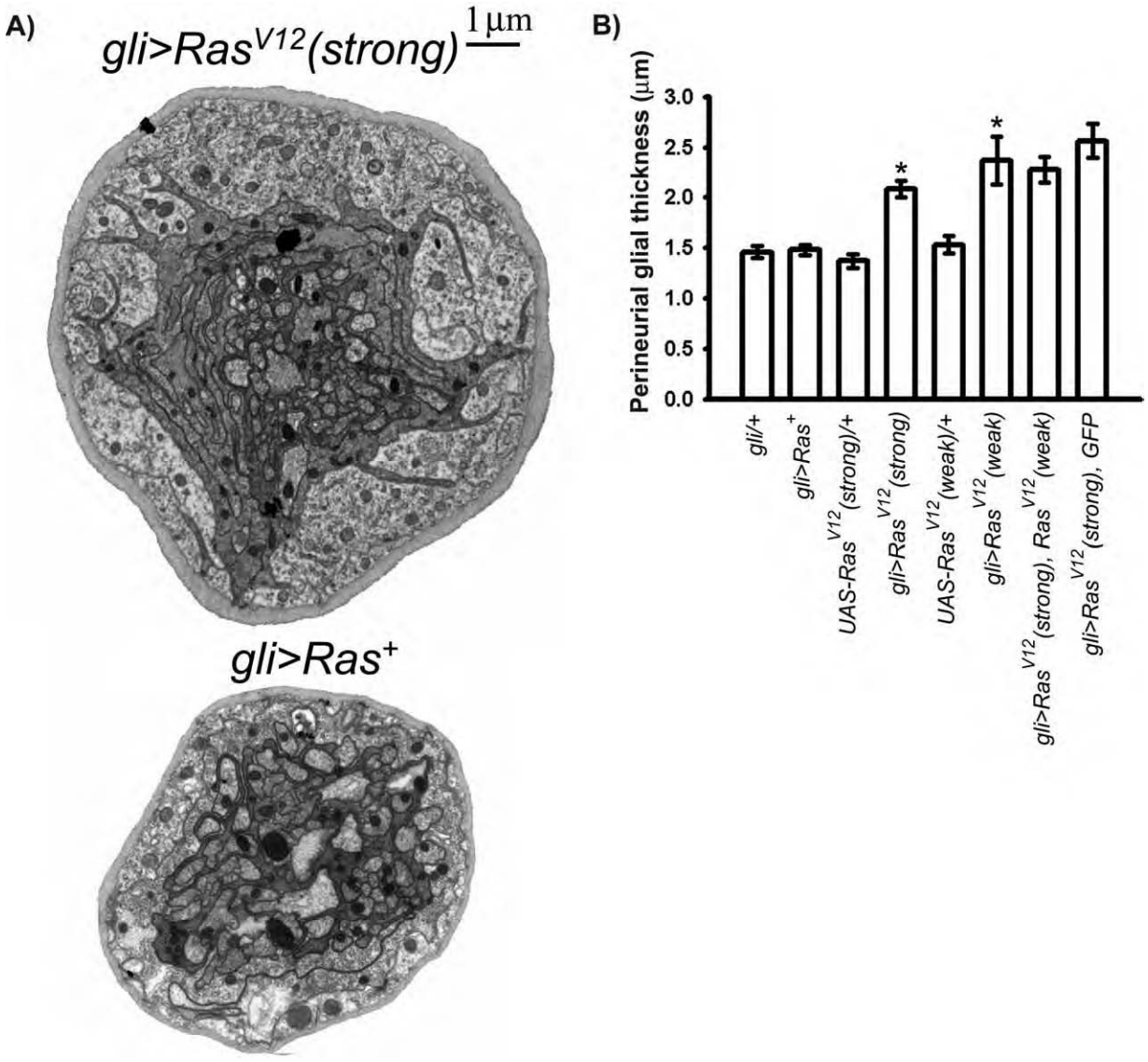
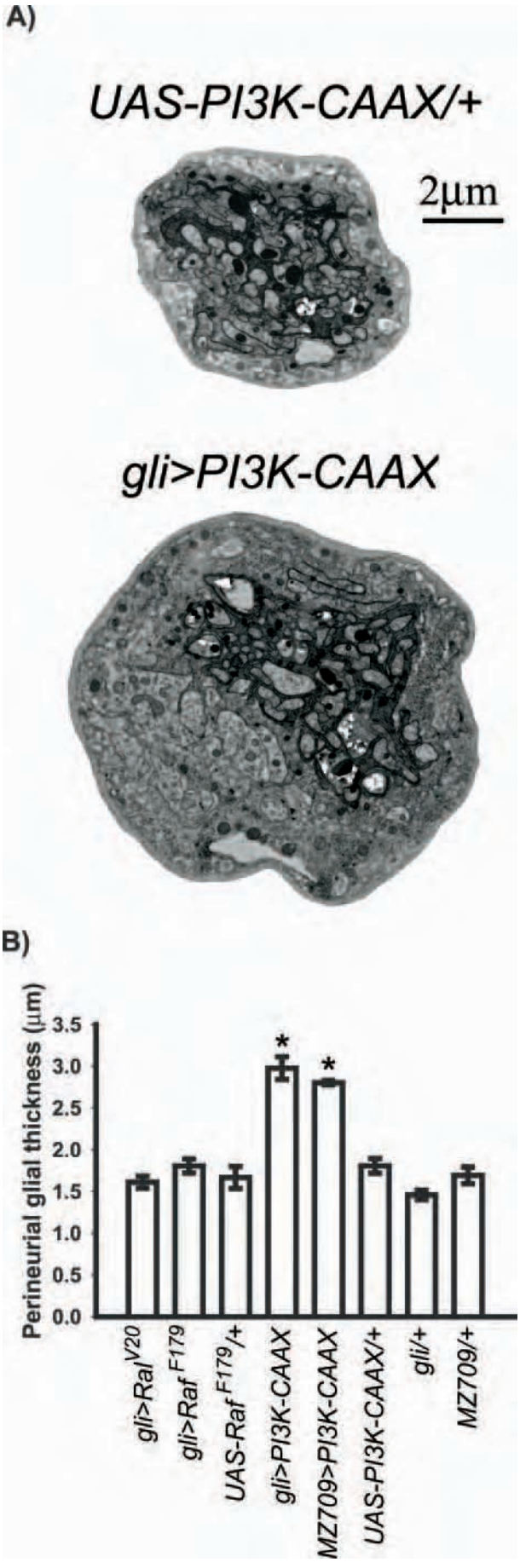


Figure 2. Expression of activated Ras in peripheral glia increases perineurial glial growth. ***A***, TEMs of cross sections from representative peripheral nerves of the indicated genotypes. The *gli>Ras*$^{+}$ nerve is the same nerve cross section shown Figure 1 *A*. ***B***, Perineurial glial thickness (*y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli>Ras*^{V12}*(strong)* (2.08 ± 0.082 μm; $n = 78$), *gli>Ras*^{V12}*(weak)* (2.37 ± 0.236 μm; $n = 20$), *gli>Ras*^{V12}*(strong)*, *Ras*^{V12}*(weak)* (2.28 ± 0.130 μm; $n = 56$), and *gli>Ras*^{V12}*(strong)*, *GFP* (2.57 ± 0.17 μm; $n = 43$) versus *gli>Ras*$^{+}$ (1.48 ± 0.048 μm; $n = 99$), *gli–Gal4/+* (1.46 ± 0.057 μm; $n = 60$), *UAS–Ras*^{V12}*(strong)/+* (1.37 ± 0.068 μm; $n = 45$), and *UAS-Ras*^{V12}*(weak)/+* (1.53 ± 0.088 μm; $n = 22$). For all such tests, $p < 0.0001$.

PI3K activity in the peripheral glia is necessary to mediate the nonautonomous, growth-promoting effect of *Ras*^{V12}

The results shown in Figure 3 demonstrate that PI3K activation in peripheral glia is sufficient to increase perineurial glial growth. To determine whether PI3K activity is necessary for the nonautonomous growth-promoting effects of *Ras*^{V12}, we introduced the heteroallelic *PI3K* loss-of-function combination *PI3K*^{2H1}/*PI3K*^{A} (Halfar et al., 2001) into *gli>Ras*^{V12} larvae. This mutant combination was chosen because it decreases PI3K activity sufficiently to confer phenotypes but retains sufficient activity to permit viability to the third-instar larval stage. We found that *PI3K*^{2H1}/*PI3K*^{A} significantly suppressed the growth-promoting effects of *Ras*^{V12} (Fig. 4), which demonstrates that PI3K activity is necessary for this effect. To determine whether PI3K activity is necessary in peripheral glia rather than the perineurial glia, we blocked PI3K activity specifically in the peripheral glia by coexpressing *Ras*^{V12} with a transgene encoding the dominant-negative *PI3K*^{D954A} (Leevers et al., 1996). We found that the peripheral–glial-specific expression of *PI3K*^{D954A} blocked the growth-promoting effects of *Ras*^{V12} (Fig. 4), suggesting that PI3K activity is required in the peripheral glia to promote perineurial glial growth. In contrast, as described above, coexpressing *Ras*^{V12} with *GFP* did not suppress the growth-promoting effects of *Ras*^{V12} (Fig. 2).

To confirm that *PI3K*^{2H1}/*PI3K*^{A} suppressed the *Ras*^{V12} phenotype by decreasing PI3K activity in the peripheral glia rather than the perineurial glia, we introduced *PI3K*^{2H1}/*PI3K*^{A} into *gli>PI3K–CAAX* larvae. The extremely thick perineurial glia conferred by *PI3K–CAAX* was not significantly affected by the presence of *PI3K*^{2H1}/*PI3K*^{A} (Fig. 4); thus, the perineurial glia in the *PI3K*^{2H1}/*PI3K*^{A} mutant is fully competent to respond to growth-promoting signals from the peripheral glia, which



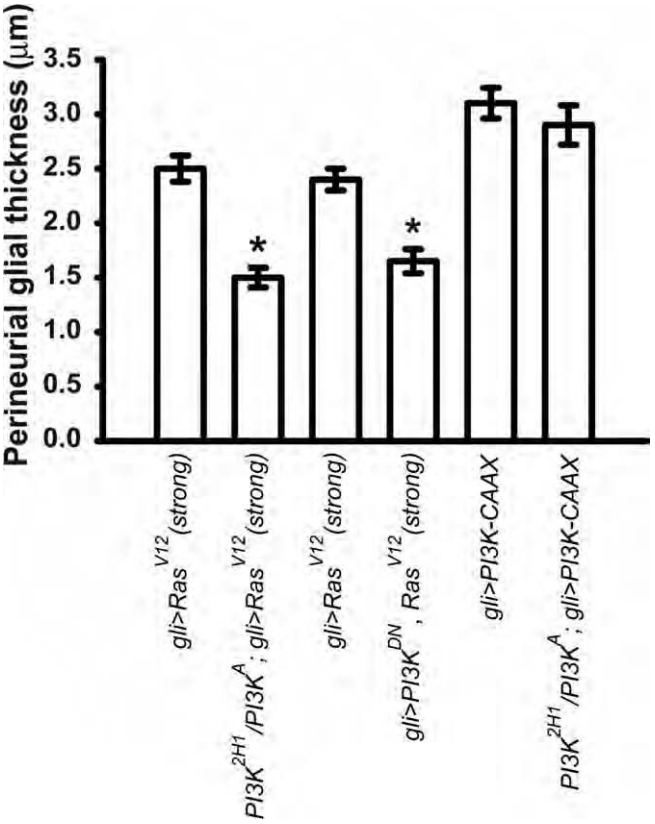


Figure 4. Ras^{V12} requires PI3K activity in the peripheral glia to increase perineurial glial growth. Histograms show perineurial glial thickness (*y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. The increase in perineurial glial thickness observed in *gli>Ras*^{V12} (lane 1) is significantly suppressed by the heteroallelic loss-of-function combination $PI3K^{2H1}/PI3K^{A}$ (lane 2). The *gli>Ras*^{V12} larvae analyzed for lane 1 were obtained from the $PI3K^{+}$ recombinants when $PI3K^{A}$ was crossed onto *UAS–Ras*^{V12}, whereas the $PI3K^{2H1}/PI3K^{A}$; *gli>Ras*^{V12} larvae analyzed were obtained from the $PI3K^{A}$ recombinants from this cross; thus, the values for the genotypes shown in lanes 1 and 2 are paired. The following pairwise combinations had statistically significant differences (two-tailed, unpaired *t* test) denoted by asterisks: *gli>Ras*^{V12}*(strong)* (lane 1; 2.46 ± 0.13 μm; $n = 50$) versus $PI3K^{2H1}/PI3K^{A}$; *gli>Ras*^{V12} (1.54 ± 0.05 μm; $n = 85$), $p < 0.0001$; for *gli>Ras*^{V12}*(strong)* (lane 3; 2.41 ± 0.111 μm; $n = 72$) versus *gli>Ras*^{V12}*(strong)*, $PI3K^{D954A}$ (1.75 ± 0.08 μm; $n = 49$), $p < 0.0001$. In contrast, *gli>PI3K–CAAX* (3.1 ± 0.14 μm; $n = 53$) was not significantly different from $PI3K^{2H1}/PI3K^{A}$; *gli>PI3K–CAAX* (2.88 ± 0.35 μm; $n = 11$), $p = 0.43$

strongly suggests that the significant suppression of the *Ras*^{V12} growth phenotype by $PI3K^{2H1}/PI3K^{A}$ results from loss of PI3K activity in the peripheral glia.

The PI3K effector Akt mediates the nonautonomous effects of PI3K on perineurial glial growth

One PI3K effector is the protein kinase Akt (Scheid and Woodgett, 2001). Elevated PI3K activity promotes the ability of

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Figure 3. Peripheral glial activity of constitutively active PI3K, but not constitutively active Ral or Raf, is sufficient to increase perineurial glial growth. ***A***, TEMs of cross sections from representative peripheral nerves of the indicated genotypes. ***B***, Perineurial glial thickness (*y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli>PI3K–CAAX* (2.98 ± 0.136 μm; $n = 76$) and *MZ709>PI3K–CAAX* (2.80 ± 0.263 μm; $n = 33$) versus *UAS–PI3K–CAAX/+* (1.81 ± 0.088 μm; $n = 59$), *gli–Gal4/+* (1.46 ± 0.057 μm; $n = 60$) and *MZ709/+* (1.69 ± 0.10 μm; $n = 27$). For all such tests, $p < 0.0001$. In contrast, *gli>Raf*^{F179} and *gli>Ral*^{V20} showed no significant increase in perineurial glial thickness.

the kinase PI3K-dependent kinase PDK1 to phosphorylate and activate Akt. To determine whether Akt activity was necessary for the growth-promoting effects of PI3K, we replaced either one or both copies of Akt^{+} with the strong hypomorphic Akt^{4226} allele (Perrimon et al., 1996) in *gli>PI3K–CAAX* larvae. We found that replacing one copy of Akt^{+} moderately suppressed, and replacing both copies of Akt^{+} profoundly suppressed, the effects of *PI3K–CAAX* (Fig. 5). These results demonstrate that Akt activity is required for the growth-promoting effects of PI3K. Akt activity can promote growth cell autonomously (Hay and Sonenberg, 2004). Thus, Akt^{4226} could suppress the growth-promoting effects of *PI3K–CAAX* by decreasing Akt activity in either the peripheral or perineurial glia. To determine whether Akt^{+} activity in the peripheral glia was sufficient to increase perineurial glial growth, we measured perineurial glial thickness in larvae expressing either of two *UAS–*Akt^{+} transgenes driven by *gli–Gal4* and found no effect on the perineurial glia (Fig. 5). Because these Akt^{+} transgenes encode wild-type Akt, which requires activation by the PI3K-dependent kinase PDK1, it was possible that this lack of effect might result from low endogenous PI3K activity in the peripheral glia, which would lead to inability to activate Akt. To test this possibility, we activated Akt in the peripheral glia by using *gli–Gal4* to coexpress *UAS–*Akt^{+} with *UAS–PI3K–CAAX*. We found a striking increase in perineurial glial thickness in this genotype compared with larvae overexpressing *PI3K–CAAX* alone (Fig. 5; note that the *gli>PI3K–CAAX,* Akt^{+} nerve pictured is an extreme nerve, not a typical nerve). This result suggests that, in the presence of activated PI3K, Akt levels within the peripheral glia become limiting for activating growth nonautonomously. In this view, increasing Akt levels by transgene expression serves to relieve this limitation and enable an additional increase in perineurial glial growth. We conclude that Akt activation in the peripheral glia is sufficient to increase perineurial glial growth.

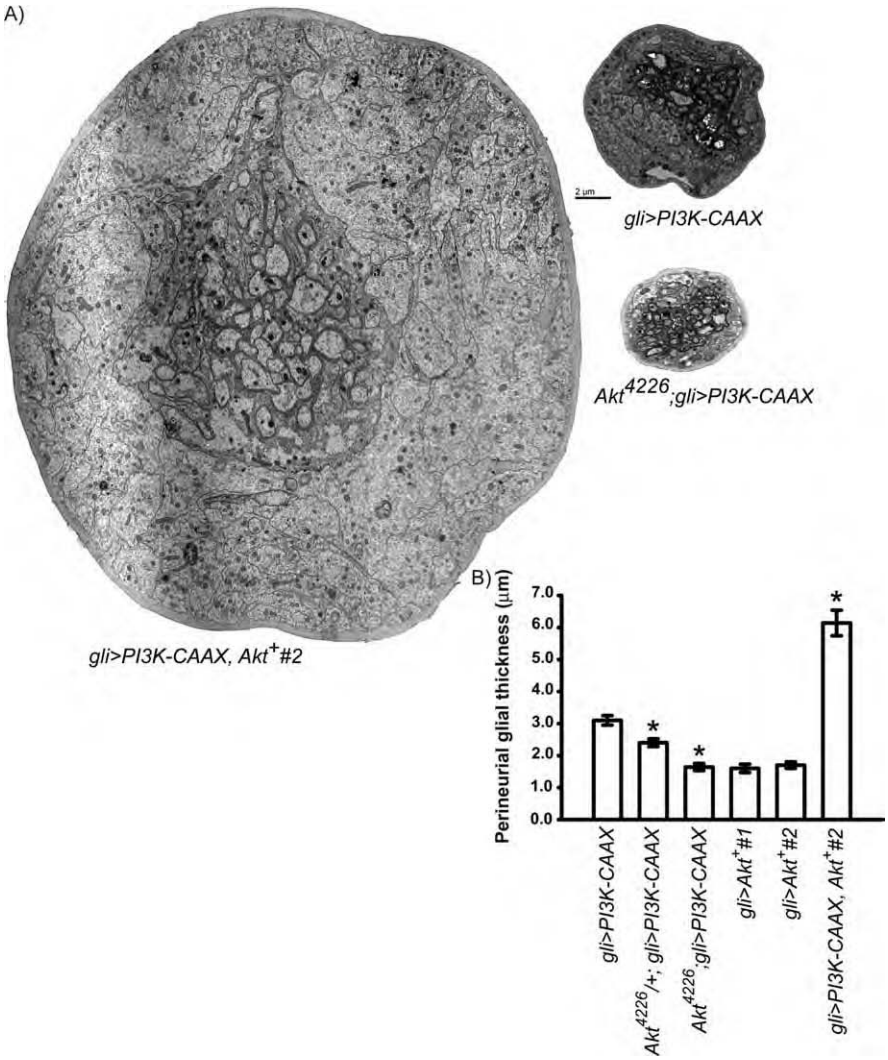
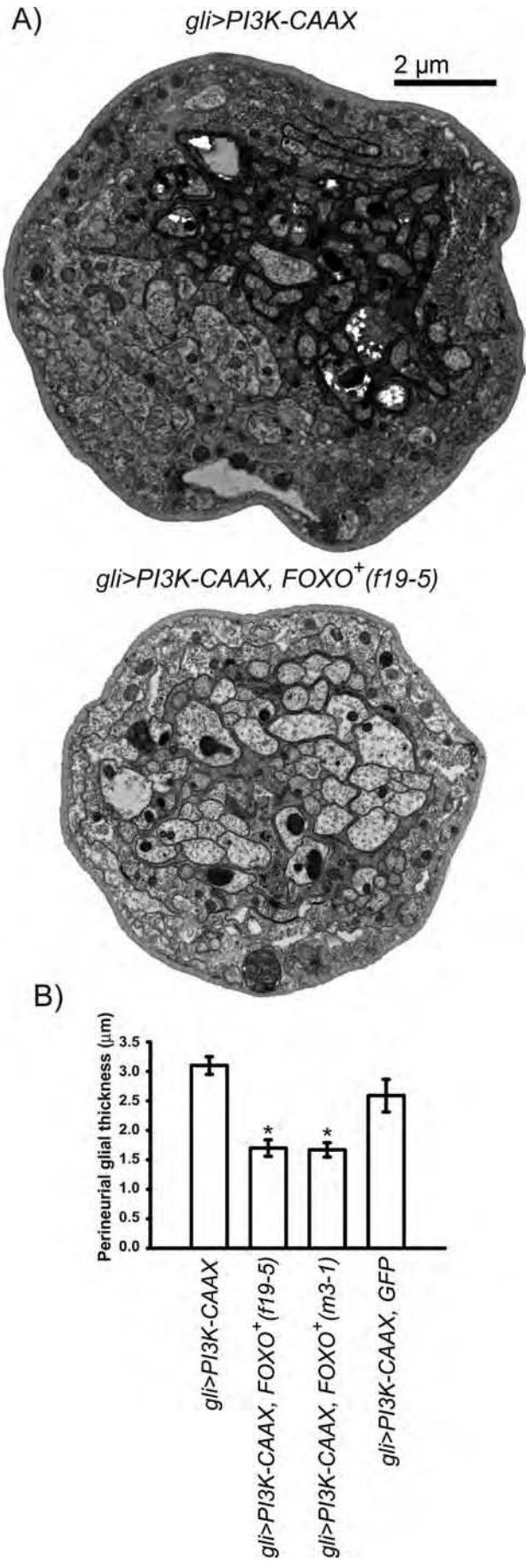


Figure 5. Akt activity in the peripheral glia is necessary and sufficient for PI3K-induced nonautomous growth activation. ***A***, TEMs of cross sections from peripheral nerves of the indicated genotypes. The *gli>PI3K–CAAX,* Akt^{+}*#2* nerve was too large to be photographed in a single electron micrograph, and thus a photo montage composed of four separate photographs is shown. This nerve is not typical and represents one of the larger nerves of this genotype. The same *gli>PI3K–CAAX* nerve from Figure 3 is shown for relative comparison with perineurial glial thickness of other genotypes. ***B***, Perineurial glial thickness (*y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli>PI3K–CAAX* (2.98 ± 0.136 μm; $n = 76$) versus Akt^{4226}*/+; gli>PI3K–CAAX* (2.42 ± 0.16 μm; $n = 29$), $p = 0.02$, and versus Akt^{4226}/Akt^{4226}*; gli>PI3K–CAAX* (1.65 ± 0.42 μm; $n = 52$), $p < 0.0001$. Also, *gli>PI3K–CAAX,* Akt^{+}*#2* (6.14 ± 0.45 μm; $n = 28$) versus *gli>PI3K–CAAX, gli>*Akt^{+}*#1* (1.48 ± 0.057 μm; $n = 32$) and *gli>*Akt^{+}*#2* (1.52 ± 0.068 μm; $n = 22$), $p < 0.0001$. *UAS–*Akt^{+}*#1* and *UAS–*Akt^{+}*#2* are independent insertions of the same transgene.

In addition to the effect of *gli>PI3K–CAAX, Akt* on perineurial glial thickness, we observed a significant increase in thickness of the "axon bundle" (motor and sensory axons and peripheral glia) in this genotype. This increased thickness is attributable mostly to the presence of motor and sensory axons of increased diameter (Fig. 5). A more complete description of this phenotype will be presented in a future study. However, these observations suggest that extremely high levels of Akt activity can nonautonomously activate axonal growth as well as perineurial glial growth.

FOXO overexpression suppresses the growth-promoting effects of PI3K

One Akt effector is the forkhead-box transcription factor FOXO. FOXO inhibits PI3K- and Akt-dependent gene expression; this activity is lost during phosphorylation by Akt, which causes phospho-FOXO to be excluded from the nucleus (Brunet et al., 1999). To test the possibility that PI3K and Akt activity increase perineurial glial growth by inhibiting FOXO, we coexpressed *PI3K–CAAX* and either of two *FOXO* transgenes (Hwangbo et al., 2004) within the peripheral glia. We found that expression of either *FOXO* transgene significantly suppressed the growth-promoting effects of *PI3K–CAAX* (Fig. 6). In contrast, when we coexpressed *PI3K–CAAX* with a neutral *UAS*-driven transgene (*UAS–GFP*), we did not observe significant suppression (Fig. 6).



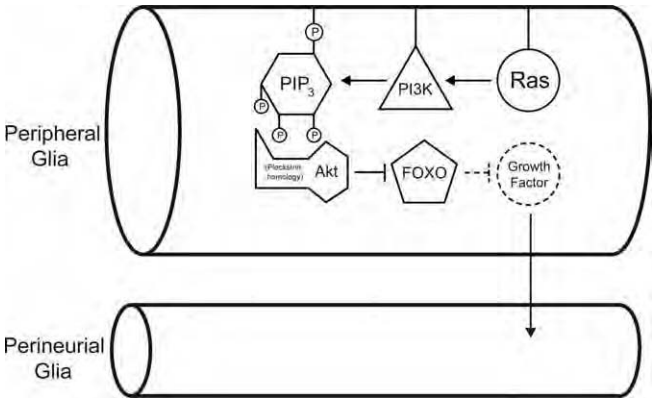


Figure 7. Model for the nonautonomous control of perineurial glial growth by the PI3K pathway. Peripheral and perineurial glial cells are indicated. The experimental evidence reported here demonstrates that Ras–PI3K–Akt activity in the peripheral glia increases growth of the perineurial glia and suggests that this activation occurs by the inhibition of FOXO (solid arrows and boxes). We hypothesize (dashed arrows and boxes) that FOXO directly or indirectly inhibits expression of a growth factor that activates perineurial glial cell growth. PIP_3, Phosphatidylinositol (3,4,5)-trisphosphate; P, phosphate.

Thus, *FOXO* overexpression suppresses the growth-promoting effects of PI3K.

Our studies provide new mechanistic insights into the nonautonomous growth-promoting effects of peripheral glia (Schwann cells) in peripheral nerves. Our results are completely consistent with the possibility that these nonautonomous effects are mediated by a pathway in which the negative regulation of growth by FOXO is inhibited by its Akt-dependent phosphorylation. FOXO might directly or indirectly repress transcription of growth factors that recruit the growth of neighbors.

Discussion

We report the effects of altered activity of Ras and downstream effectors on growth within *Drosophila* peripheral nerves. We found that activating Ras specifically within the peripheral glia was sufficient to increase growth of the perineurial glia. In addition, we found that activating the Ras effector PI3K (Rodriguez-Viciana et al., 1994), but not Raf or Ral, within the peripheral glia was sufficient to increase perineurial glial growth and that inhibiting PI3K activity in the peripheral glia, but not perineurial glia, suppressed the growth-promoting effects of activated Ras. We also found that activity within the peripheral glia of the PI3K-activated kinase Akt (Franke et al., 1995; Scheid and Woodgett, 2001) was both necessary and sufficient to mediate the growth-promoting effects of PI3K. Finally, we found that overexpression within the peripheral glia of FOXO, the forkhead-box transcription factor that is phosphorylated and inactivated by Akt-dependent phosphorylation (Brunet et al., 1999), was sufficient to suppress the growth-promoting effects of PI3K. Together,

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Figure 6. PI3kinase and Akt increase perineurial glial growth by inhibition of FOXO. ***A***, TEMs of cross sections from peripheral nerves of the indicated genotypes. The same *gli–PI3K–CAAX* nerve from Figure 3 is shown for relative comparison of perineurial glial thickness to *gli>PI3K–CAAX, FOXO+ (f19-5)* nerve. ***B***, Perineurial glial thickness (*y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli>PI3K–*CAAX (2.98 ± 0.136 μm; $n = 76$) and *gli>PI3K–CAAX, GFP* (2.59 ± 0.28 μm; $n = 25$) versus *gli>PI3K–CAAX, FOXO+ (f19-5)* (1.67 ± 0.086 μm; $n = 30$), $p < 0.004$, and versus *gli>PI3K–CAAX, FOXO+ (m3-1)* (1.70 ± 0.098 μm; $n = 64$), $p < 0.01$. *UAS–FOXO+ (f19-5)* and *UAS–FOXO+ (m3-1)* are independent insertions of the same transgene.

these results suggest that Ras activity in the peripheral glia activates nonautonomous growth via the PI3K and Akt-dependent inhibition of FOXO (Fig. 7). This observation is consistent with the previous observations that *Nf1*$^-$ mouse Schwann cells oversecrete growth factor(s) that cause increased recruitment of mast cells into the peripheral nerve (Yang et al., 2003) and is consistent in part with the observation that the proliferation defects of *Nf1*$^-$ mutant mouse or human cells requires hyperactivation of Tor in a PI3K- and Akt-dependent manner (Dasgupta et al., 2005; Johannessen et al., 2005).

Regulation of peripheral nerve growth by a neuron–glia signaling pathway

Yager et al. (2001) reported that perineurial glial growth in *Drosophila* peripheral nerves is regulated by several genes. These genes include *Nf1*, which is the *Drosophila* ortholog of human *Nf1*, *push*, which is thought to encode an E3 ubiquitin ligase and two genes implicated in neurotransmitter signaling: *amnesiac*, which is thought to encode a neuropeptide similar to vertebrate pituitary adenylate cyclase-activating polypeptide (Feany and Quinn, 1995), and inebriated (*ine*), which encodes a member of the Na^+/Cl^--dependent neurotransmitter transporter family (Soehnge et al., 1996). Some of these genes might regulate perineurial glial growth via the activity of Ras or PI3K within peripheral glia. For example, mutations in *push*, but not *ine*, enhance the perineurial glial growth phenotype of *Ras*^{V12} expressed in peripheral glia (Lavery and Stern, unpublished observations). These observations are consistent with the possibility that the activity of *ine* regulates Ras-GTP levels within peripheral glia. In contrast, *push* might regulate PI3K in a Ras-independent manner or act in the perineurial glia to regulate sensitivity to peripheral glial growth factors. Additional experiments will be required to distinguish between these possibilities.

Regulation of peripheral nerve growth by Schwann cell nonautonomous mechanisms

There are several lines of evidence from mice and humans suggesting that cell nonautonomous growth regulation, as a consequence of intercellular signaling, underlies neurofibroma formation. First, although neurofibromas arise in individuals heterozygous for *Nf1*$^-$ after loss of *Nf1*$^+$ from cell(s) within peripheral nerves, neurofibromas are heterogeneous and contain cells that are not clonally related, such as Schwann cells, perineurial cells, and fibroblasts. These observations suggest that neurofibromas arise when a core of *Nf1*$^-$ cells cause overproliferation of their heterozygous neighbors via nonautonomous means. Second, neurofibroma formation in mice and humans requires a homozygous *Nf1* mutant genotype in Schwann cells but not other cells within the tumor (Kluwe et al., 1999; Zhu et al., 2002). Third, Ras-GTP levels in Schwann cells from the mouse *Nf1* knock-out mutant are uniformly elevated. In contrast, only a subset of Schwann cells from human neurofibromas exhibit elevated Ras- GTP levels (Sherman et al., 2000); these authors raised the possibility that this subset, but not other Schwann cells from the tumor, was homozygous for *Nf1*$^-$. In this view, these *Nf1*$^-$ cells recruited neighboring Schwann cells that were heterozygous for *Nf1*$^-$ into the tumor by nonautonomous means, such as by the excessive release of one or more growth factors. Fourth, Yang et al. (2003) demonstrated that *Nf1*$^-$ Schwann cells oversecrete the ligand for the c-Kit receptor. This oversecretion increased migration of mast cells into peripheral nerves and might be an essential step in neurofibroma formation. These Schwann cells also oversecrete additional factors whose physiological role remains unclear (Yang et al., 2003). The molecular mechanisms by which neurofibromin regulates the synthesis or release of these molecules remain incompletely understood. Our observations that Ras activity in the peripheral glia promotes growth nonautonomously via the PI3K- and Akt-dependent inhibition of FOXO might provide insights into the mechanisms by which peripheral nerve growth is regulated nonautonomously by the mammalian Schwann cell.

Regulation of peripheral nerve growth by Ras effectors

By hyperactivating Ras, *Nf1* mutations could in principle cause tumors via any of several Ras effector pathways. In addition, the diverse types of tumors observed in individuals with neurofibromatosis (for review, see Cichowski and Jacks, 2001) could result from hyperactivation of distinct Ras effector pathways. The Raf pathway has been viewed previously as a more relevant effector pathway than the PI3K pathway, mostly because the importance of Ras in the activation of PI3K under physiological conditions remains controversial. In particular, although it is clear that the oncogenic *Ras*^{V12} mutant is sufficient to activate PI3K (Rodriguez-Viciana et al., 1994), it has sometimes been difficult to demonstrate that wild-type Ras is necessary for PI3K activation (Prober and Edgar, 2002). Presumably, this difficulty reflects the fact that PI3K can be activated by Ras-independent as well as Ras-dependent mechanisms, such as direct activation by activated receptor tyrosine kinases or by PIKE-L (phosphatidylinositol kinase enhancer) (Escobedo et al., 1991; Rong et al., 2004). However, more recently, it has been demonstrated that PI3K and Akt are hyperactivated in several *Nf1* mutant cell types and that this hyperactivation is Ras dependent (Dasgupta et al., 2005; Johannessen et al., 2005). Furthermore, PI3K activation plays an essential functional role in *Nf1*$^-$-mediated growth defects, as was demonstrated by the observation that PI3K- and Akt-dependent Tor activation was necessary for the proliferation defects of *Nf1* mutants to occur: application of rapamycin, a Tor inhibitor, attenuated the ability of *Nf1* mutant cells to proliferate (Johannessen et al., 2005). These observations demonstrate that PI3K and Akt play key roles in at least some aspects of *Nf1*$^-$-induced tumor growth.

Our results are consistent with these observations. By comparing the effects on perineurial glial growth of peripheral–glial expression of activated *Raf*, *PI3K*, or *Ral*, we were able to demonstrate that activation of PI3K, not Raf or Ral, was sufficient to promote perineurial glial growth and that PI3K activity in the peripheral glia was necessary to observe the nonautonomous effect of activated Ras on perineurial glial growth. We similarly showed that Akt activity was necessary and sufficient to mediate the growth-promoting effects of PI3K. However, whereas Dasgupta et al. (2005) and Johannessen et al. (2005) observed that Tor activation was critical for the PI3K- and Akt-dependent growth regulation of *Nf1* mutant cells, we observed a critical role for the PI3K- and Akt-dependent inhibition of the transcription factor FOXO. It is possible that the phenotype observed by Dasgupta et al. (2005) and Johannessen et al. (2005) reflects the well characterized ability of PI3K–Tor to activate growth cell autonomously (Hay and Sonenberg, 2004), whereas the phenotype we report reflects nonautonomous growth regulation. In this view, PI3K and Akt regulate autonomous and nonautonomous growth via the Tor and FOXO pathways, respectively.

FOXO presumably inhibits the growth-promoting effects of PI3K and Akt by transcriptional regulation of target genes. Candidate FOXO target genes include those encoding the molecules oversecreted by *Nf1*$^{-}$ Schwann cells (Yang et al., 2003), whereas other targets might be represented in the distinct transcript profiles exhibited by *Nf1*$^{-}$ Schwann cells (Mashour et al., 2001) or malignant peripheral nerve sheath tumors (Miller et al., 2006) compared with wild-type Schwann cells. For example, Schwann cells from neurofibromas, but not normal Schwann cells, express the epidermal growth factor (EGF) receptor (DeClue et al., 2000). Other potential targets include genes encoding growth factors, although ectopic expression within the peripheral glia of two candidate genes, *Hedgehog* and the EGF ligands *spitz* and *gurken*, failed to induce perineurial glial growth (Lavery and Stern, unpublished observation). Additional experiments will be required to identify the FOXO targets that regulate nonautonomous growth in peripheral nerves.

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